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Local and systemic antibody responses and immunological memory in humans after immunization with cholera B subunit by different routes

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A single oral or intramuscular immunization with purified cholera B subunit induced an intestinal secretory immunoglobulin A (IgA) antitoxin response in, respectively, 10 out of 11 and 9 out of 12 Bangladeshi volunteers. The IgA titre rise in intestinal lavage fluid was similar by either route of immunization, but the duration of the response was usually longer after the oral dose. A second immunization by either route, given 25 days after the first, and a third dose (oral only), given 15 months later, resulted in intestinal immune responses which did not differ in magnitude from that induced by the initial immunization but were observed significantly earlier, usually by day 3. Both the first oral and intramuscular immunizations induced significant antitoxin titre rises, mainly IgG, in the serum in most vaccinees but the magnitude of the response was considerably higher after the intramuscular dose. Significant IgA antitoxin titre rises in saliva and breast milk were seen after both oral and intramuscular immunization.

Unlike clinically manifest cholera, which seems to give considerable protection against another attack of the disease for several years (*1*, *2*), parenteral whole-cell cholera vaccines offer only partial protection for less than 6 months (*3*). This relatively poor efficacy of cholera vaccines may be due to deficient composition of the immunogen or to inefficient administration of the vaccine, or to both.

Previous studies in animals have shown that antibacterial and antitoxin antibodies cooperate synergistically in protecting against experimental cholera in the gut (*4*), suggesting that an optimum cholera vaccine should contain toxin-derived as well as somatic antigens. The nontoxic pentameric B subunit portion of cholera toxin (*5*) appears to be a very suitable "toxoid" component of such an improved cholera vaccine. Though probably less immunogenic than the cholera holotoxin (*6*), the B subunit portion is a good immunogen, much better than the toxin-active cholera A subunit; antibodies to the B subunit also have a much stronger cholera-toxin-neutralizing capacity than anti-A antibodies (*7*, *8*). Furthermore, the fact that the B subunit has retained the ability to bind specifically to the intestinal mucosa seems to make it especially apt for oral immunization (*5*, *6*).

The noninvasive nature of cholera infection and the localized action of cholera toxin on the lining epithelial cells of the small intestine preclude a protective effect of systemic immune factors unless these have diffused from the circulation into the gut. It is most likely therefore that immune protection against cholera is mainly, if not exclusively, mediated by locally produced secretory IgA (SIgA) antibodies. In animal studies, such gut mucosal secretory immunity has been evoked by either parenteral or oral antigen stimulation but, in general, the oral route has been found to be more effective (*6*, *9*, *10*). In humans, clinical cholera has been shown to give rise to antitoxin as well as antibacterial SIgA antibody formation in the intestine although the observed titre increases have generally been of short duration (*11*). Therefore, it is possible that the long-lasting immunity to second attacks of cholera seen both in volunteers (*1*) and in an endemic area (*2*) might be the result of rapid, efficient boosting of anamnestic antibody formation by the gut from cholera antigen stimulation due to renewed infection rather than to

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continuing antibody formation after the initial antigen stimulation; in endemic areas the gut mucosal system may receive many repeated booster stimuli from natural exposure to cholera antigens.

The aim of the present study was to evaluate the local and the systemic immunogenicity of purified B subunit of cholera toxin given by different routes and to explore the capacity of this immunogen to induce an immunological memory, particularly in the intestine. Based on recent studies suggesting that immune responses in the intestine may be reflected in other secretions (*12, 13*), antibody responses to B subunit were studied in breast milk and saliva as well. A preliminary report describing some of the gut mucosal responses has been published (*1, 4*).

MATERIALS AND METHODS

Cholera B subunit. Purified B subunit of cholera toxin was prepared by the Swedish National Bacteriological Laboratory in accordance with the European Pharmacopoeia recommendations for human vaccine production using the procedure previously described (*15*). The B subunit preparation was diluted in physiological saline to 0.5 mg/ml, merthiolate was added to 0.01% final concentration and the final preparation, after sterile filtering and dispensing in 1 ml vials, stored at 4 °C until used. The preparation lacked toxicity in mice and guinea pigs in tests after oral, intraperitoneal and intravenous administration of up to 5 ml doses according to the requirements of the European Pharmacopœia, and was found to contain no detectable toxic activity in rabbit skin (*16*) and small bowel loops (*17*) when tested in amounts 10 000 times higher than the minimal effective dose of holotoxin in the respective assays. The purity of the B subunit portion was demonstrated by means of SDS gel-electrophoresis (*18*), immunodiffusion, and ELISA tests using antiserum to *Vibrio cholerae* concentrated culture filtrates (*19*). The ganglioside GM1-binding capacity of the cholera B subunit was similar to that of the holotoxin (*20*) and the protective immunogenicity in rabbits (*15*) was only slightly less than that of intact cholera toxin.

Immunization groups. Twenty-four healthy women in the mature lactation phase, 2–10 months after delivery, and 6 non-lactating women were studied. Their ages varied between 20 and 35 years and they weighed between 24 and 42 kg. A signed informed consent was obtained from the 30 subjects who were then carefully examined by a physician in order to exclude from the study any person with a history or signs of pulmonary or cardiac failure, glaucoma, tuberculosis, diabetes, increased intracranial pressure, or diarrhoeal disease that required hospitalization during the previous 12 months, or who showed a positive pregnancy test; only 1 person (out of 31 initially recruited) had to be excluded because of a positive pregnancy test. The 24 lactating women were divided into four groups of 6 persons and all were given 2 doses of cholera B subunit 25 days apart by either the oral or intramuscular route, as shown in Table 1. Fifteen months later, 16 of these women (all those who could be contacted) were given a third oral immunization with 500 μg of cholera B subunit in a single dose. Before each oral immunization, 150 ml of an 0.1 mol/l $NaHCO_3$ solution was given as a drink to neutralize gastric acidity and 5 minutes later the B subunit dose was given in 150 ml of the same solution.

Table 1. Immunization groups for receiving oral and intramuscular doses of cholera B subunit

Group	First dose	Second dose[a]	Third dose[b]
I	Oral[c]	Oral	Oral
II	Intramuscular[d]	Intramuscular	Oral
III	Oral	Intramuscular	Oral
IV	Intramuscular	Oral	Oral

[a] Interval between first and second doses was 25 days.
[b] Interval batwaan sacond and third dosas was 15 months.
[c] Each oral dose contained 500 μg of cholera B subunit.
[d] Each intramuscular dose contained 150 μg of cholera B subunit.

Toxicity surveillance. Following each administration of cholera B subunit, a physician examined the vaccinees daily for ten days. Local and systemic side-effects were recorded on vaccine surveillance forms; the reactions after parenteral immunization were graded from 0 to 4 (no reaction to pronounced), and after the oral immunizations as either 0 (absent) or + (present).

Sampling of specimens. Intestinal lavage fluid, serum, saliva and in most instances also breast milk and stool specimens were collected on the same day as, or the day before ("day 0"), the first immunization and then 7, 14, and 25 days after the first and 7, 14 and 28 days after the second immunization, and then again on day 0, 3, 9, and 28 after the third immunization.

The intestinal lavages were performed by allowing the subjects to drink 250 ml of an isotonic salt solution every 10 minutes until a watery diarrhoea resulted, and then continuing with this intake of fluid until 1000 ml of liquid stool was collected. The liquid stool was then heat-inactivated, Millipore-filtered, centrifuged, and concentrated as previously described (*11, 14*). The first formed stool obtained from each

woman after initiating the lavage procedure was collected separately, dissolved by mechanical agitation in phosphate-buffered saline (PBS, 0.01 mol/l phosphate, 0.15 mol/l NaCl, pH 7.2), heat-inactivated at 56 °C for 15 min, homogenized using a wooden stick, and centrifuged at 10 000 *g* for 15 min to remove insoluble material.

Serum was prepared from a fingerstick specimen of 200 µl of blood diluted in 1.8 ml of sterile saline. Whole saliva was collected after stimulation by letting the women chew on parafilm (laboratory film) until they had produced 5 ml of saliva; the salivary specimens were then heated at 56 °C for 15 min and centrifuged at 5000 *g* for 15 min. Milk specimens were expressed manually from the breasts and centrifuged at 5000 *g* for 15 min, after which the clear middle layer was collected. All specimens were divided into 3 different vials and kept frozen at − 70 °C (in the case of lavage and stool specimens) or at − 20 °C (serum, breast milk and saliva) until examined. One vial of each frozen specimen was dispatched by air to Sweden and if it thawed during transport, it was immediately refrozen on arrival 1–2 days later.

Antigens. Highly purified cholera toxin was purchased (Schwarz/Mann, Orangeburg, PA, USA), while purified A subunit was prepared as previously described (*15*) and the B subunit was the same preparation as used for immunization. Highly purified *V. cholerae* lipopolysaccharide was prepared from the classical strain 35A3 (Inaba) by extraction with hot phenol water followed by repeated ultracentrifugation (*21*).

Antibody determinations. Antitoxin antibodies of different classes were determined by means of ELISA (*14*, *19*) in polyvinyl (Cooke, Alexandria, VA, USA) or polystyrene microtitration plates (Dynatech, Plochingen, Federal Republic of Germany), which had been precoated with 0.1 ml (per well) of a 2 µg/ml solution of cholera toxin in PBS at 37 °C overnight; in some instances, the plates were coated with 0.1 ml (per well) of 2 µg/ml of A subunit or B subunit of cholera toxin or with 50 µg/ml of purified *V. cholerae* Inaba lipopolysaccharide instead (*19*). After washing the coated plates three times in PBS containing 0.05% Tween 20, 5-fold serial dilutions of the test specimens (0.1 ml volumes) diluted in PBS-Tween were incubated in the coated wells at room temperature overnight and, after washing, alkaline phosphatase-conjugated antihuman-IgG, -IgA or -IgM (Orion, Helsinki, Finland), diluted 1/100 in PBS-Tween, was added and allowed to react with the bound antibodies for 2 h. After renewed washing, the enzyme substrate *p*-nitrophenyl phosphate, diluted to 1 mg/ml in 1 mol/l ethanolamine buffer at pH 9.8, was added (0.1 ml/well) and, after reaction for 100 min at room temperature, the yellow colour reactions were recorded spectrophotometrically. Antibody titres were determined as the interpolated dilution of the test sample, giving an absorbance value at 405 nm of 0.4 above the background when the test wells were allowed to react for 100 min with the *p*-nitrophenyl phosphate substrate; this high absorbance value was chosen to make sure that the specific absorbance was on the steep slope of the titration curve (*19*), thereby avoiding the risk of false positive titres. The titres obtained by interpolation were then adjusted in relation to a reference specimen included in each plate to compensate for any variations between experiments. A pool of Dhaka colostral specimens (PDC) served as the IgA antibody reference material and a pool of Bangladeshi cholera convalescent sera (NIH, reference serum was provided by the National Institutes of Health) as the IgG antibody reference material. The PDC was assigned an ELISA antitoxin IgA titre of 300 000 ELISA units/ml based on a mean titre of 1/30 000 for 0.1 ml volume determined in 50 repeated ELISA tests of this specimen; correspondingly, the NIH serum was assigned an ELISA antitoxin IgG titre of 100 000 ELISA units/ml and an antitoxin IgM titre of 30 000 units/ml. The ELISA titre ascribed to each sample was the mean of 2–4 determinations.

Total IgA and total IgG were measured with an immunobead ELISA (*22*), using immunobead-adsorbed anti-IgA or anti-IgG (BioRad, Richmond, CA, USA) as catching antibody. In some instances, total IgA was also determined with a microplate ELISA method. This modified ELISA was identical with the antitoxin assay except that goat antihuman IgA (BioRad Laboratories, Richmond, CA, USA), diluted 1/5000, rather than cholera toxin was used to precoat the microplates at 4 °C for 24 h. Specific antibody activities in the different secretions were calculated by dividing the ELISA antitoxin levels with the total IgA or IgG concentration (µg/ml) of the sample.

The specificity of the ELISA determinations was ascertained by titrating a number of specimens in parallel on cholera-toxin-coated and non-coated plastic trays. It was found that particularly the lavage specimens gave some unspecific binding to non-coated plates but always less than 25% of the binding observed in the cholera-toxin-coated plates; subtraction of the absorbance values obtained in the non-coated wells from the absorbance seen in the cholera-toxin-coated wells did not change the titre values.

The range of reproducibility (coefficient of variation) of the ELISA titres, after correction to the reference specimen included in each plate, was ± 13%; and that of the antibody activities in secretions, i.e., the mean of two antitoxin titrations

divided by the total Ig value of the sample, was ±14%. Based on this variation, a 1.4-fold titre increase in antibody activity between pre- and post-immunization samples is statistically significant ($P < 0.01$); in the present study, however, we stipulated a 2-fold or greater difference between day-0 and postimmunization specimens to indicate a significant antibody rise or fall. Cholera-toxin-neutralizing antibodies were assayed by means of the adrenal cell test as previously described (*23*). The neutralizing titres given have been adjusted in relation to a reference anticholera toxin antiserum containing 4470 antitoxin units/ml (Swiss Serum Vaccine Institute, Berne, Switzerland) included in each experiment. A 4-fold or greater titre rise between pre- and post-immunization samples was regarded as significant.

Determination of the proportion of secretory IgA antibodies. The proportion of IgA antibodies that was of the SIgA class was determined by titrating the specimens by the IgA-specific ELISA method before and after passage through a column containing anti-secretory component immunoglobulin covalently coupled to sepharose, using procedures that have been described before (*13*).

RESULTS

Side-effects. In no instance did oral immunization with cholera B subunit give any detectable local or systemic side-effects whatsoever. After the intramuscular injections also systemic reactions were completely absent, although very mild pain and tenderness were found at the injection site in about half of the vaccinees; in a few instances a slight local redness and/or induration was found as well. The local side-effects either appeared early (on days 1–3), or late (on days 4–10) after the immunization; in some women a biphasic pattern was recorded with symptoms both early and late.

Intestinal antibody responses. The intestinal antibody response to immunization was studied in the intestinal lavages and in faecal specimens. Since the total Ig concentration in intestinal lavages varied considerably both between different individuals and between different samples from the same person, all titres were adjusted in relation to the total Ig of the corresponding class. A single oral immunization induced significant (i.e., ⩾2-fold) intestinal lavage ELISA IgA antitoxin titre rises in 10 out of 11 (91%) of the lactating women (Table 2); in 8 of these 10 responders the increase was ⩾4-fold. After a single intramuscular injection ⩾2-fold IgA antitoxin titre rises were seen in 9 out of 12 women (75%) and ⩾4-fold rises in 7 of these 9 responders. Most of the women responded with a significant titre increase within 1 week of the oral as well as intramuscular immunization (Table 2); significant peak IgA antitoxin titres were attained as early as on day 7 in 60% of the orally and in 33% of the intramuscularly vaccinated women (data not shown). The magnitude of the responses by the two routes was comparable, the geometric mean titre increase being 9-fold after the oral dose and 8-fold after the intramuscular

Table 2. Number and percentage of significant (i.e., ⩾2-fold) antitoxin IgA increases in relation to total IgA in intestinal lavage specimens at different time intervals after first, second and third immunizations with cholera B subunit given orally or intramuscularly

Number of days after immunization	First immunization		Second immunization[a]				Third immunization[a]
			Oral		Intramuscular		
	Oral ($n = 11$)	Intramuscular ($n = 12$)	After first oral ($n = 6$)	After first intramuscular ($n = 7$)	After first oral ($n = 5$)	After first intramuscular ($n = 5$)	Oral[b] ($n = 15$)
3	–	–	–	–	–	–	9 (60)[c]
7–9	10 (91)[c]	7 (58)	4 (67)	4 (57)	4 (80)	2 (40)	12 (80)
14	7 (64)	6 (50)	3 (60)[d]	3 (42)	4 (80)	0	–
25–28	7 (64)	3 (25)	3 (60)[d]	3 (42)	2 (40)	1 (20)	9 (60)
Up to 28	10 (91)	9 (75)	5 (83)	5 (72)	5 (100)	3 (60)	13 (87)

[a] Interval between 1st and 2nd immunization was 25 days, and between 2nd and 3rd immunization was 15 months.

[b] The third immunization was oral, the preceding two immunizations being two orals, or oral followed by intramuscular, or intramuscular followed by oral, or two intramuscular doses.

[c] Figures in parentheses are percentages.

[d] Only specimens from 5 persons were available on these days

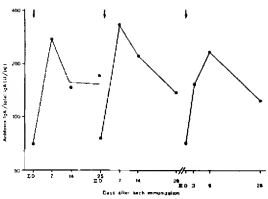

Fig. 1. The IgA antitoxin antibody responses, expressed as geometric means of IgA antitoxin titres per total amount of IgA, in intestinal lavage fluid after first (I), second (II), and third (III) oral immunizations with cholera B subunit. The arrows indicate the day of immunization.

injection; these responses were similar to that (15-fold) induced by a single oral immunization in the non-lactating women. However, the duration of the response was usually longer after the oral than after the intramuscular immunization.

The IgG antitoxin levels in the lavage specimens were also increased by either route of immunization (data not shown). Although the magnitudes of the responses (antitoxin IgG per total IgG) were comparable to those seen for IgA antitoxin, the concentration of total IgG was about 10-fold lower than that of total IgA, the geometric mean values being 2 μg/ml for IgG and 20 μg/ml for IgA. This means that the overall IgG antitoxin response was only about 10% of that of IgA.

A second oral dose given 25 days after the initial immunization resulted in IgA antitoxin responses of similar frequency (Table 2) and magnitude (Fig. 1) as the first oral dose; this was true when the second oral dose followed either an initial oral or intramuscular immunization. A second dose by intramuscular injection gave rise to a higher magnitude and frequency of responses when it followed an initial immunization given orally, than when it followed an initial intramuscular injection. Peak responses were observed within 7 days after the second oral or intramuscular immunization in 12 out of 23 of the test subjects; in 4 of the remaining 6 responders the highest antitoxin antibody levels were reached 14 days after the second dose.

Significantly raised IgA antitoxin levels were found in only 27% of the subjects 4 weeks after either of the two intramuscular injections, but in 57% of the subjects 4 weeks after the initial or second oral antigen administration (Table 2). Fifteen months later, however, only 1 out of 16 women had a higher level of IgA antitoxin in the intestine compared with the levels after the initial administration of B subunit.

The third immunization given orally 15 months later also resulted in significant ($\geq$ 2-fold) IgA antitoxin increases in the intestinal lavage in 13 out of 15 women (Table 2); in 8 of the 13 responders the titre rise was $\geq$ 4-fold. As in the case of the second immunization, the magnitude and duration of the response to the third dose did not exceed those induced by the first oral antigen administration (Fig. 1). However, contrary to the findings in a concurrent study in other women from the same village in which a primary administration of cholera B subunit failed to induce significant IgA antibody responses within the first 3 days (*24*), a significant IgA antitoxin increase was observed by day 3 in most of the women given the late oral immunization (Table 3). This early response after the third oral dose resembles that seen in a parallel study after a second oral dose of cholera B subunit given 4 weeks after the initial B subunit immunization (Table 3) (*24*).

The content of toxin-neutralizing antibodies in the lavage specimens could not be determined owing to different levels of toxin-binding material (a heat-stable non-immunoglobulin) in the lavages. By titrating the lavage specimens for IgA antitoxin antibodies in the ELISA before and after passage through a column with anti-human secretory component coupled to sepharose, it was found that, on average, 86% of the IgA antitoxin in lavages collected from orally immunized women were of the secretory type, i.e., they were locally produced. The corresponding figure for lavage specimens from intramuscularly immunized women was 71%.

Table 3. Frequency of significant intestinal IgA antitoxin responses by day 3 after a first, a second, and a third oral immunization with cholera B subunit

Immunization	Frequency of significant responders
Initial	1/16[a]
Second (25 days later)	10/16[a] ($P < 0.05$)[b]
Third (15 months after the second immunization)	9/15 ($P < 0.05$)[b]

[a] Data from Svennerholm et al. (*24*) Eight women were given 0.5 mg of B subunit and eight others were given 2.5 mg of B subunit orally as the initial immunization; 25 days later all of them were given a second oral immunization with 0.5 mg of B subunit.

[b] Comparison with the frequency of responses after the initial immunization.

Intestinal antibodies in faecal homogenates were also analysed. In many samples neither any antitoxin nor total IgA could be detected, and in the other samples the levels were generally low and showed marked variations that did not relate meaningfully to the immunization schedules. It was concluded that the faecal specimens were not useful for studying gut mucosal immune responses.

Antibody responses in serum. A single oral immunization induced significant (i.e., ≥ 2-fold) titre increases of ELISA IgG antibodies in 11 out of 12 of the lactating women, the mean titre increase being 6.5-fold (Table 4). An intramuscular immunization gave IgG antitoxin seroconversions of the same frequency but of higher magnitude (11-fold), compared with the oral immunization. Similarly, both the oral and intramuscular immunizations resulted in significant ELISA IgA titre increases in most instances and again the mean titre increase was higher after the intramuscular immunization (Table 4). The comparatively high levels of IgM antibodies in the serum before immunization were not significantly increased either by oral or intramuscular antigen administration; these antibodies might therefore be of low specificity.

Table 4. Serum IgG, IgA, IgM and neutralizing antibody responses[a] to a single oral or intramuscular dose of cholera B subunit on days 0, 7, 14 and 25 after immunization

Immunization route and antibody	Geometric mean titres (units/ml)			
	Day 0	Day 7	Day 14	Day 25
Oral:				
ELISA IgG	11600	17000	65000	76000
IgA	2500	4900	7000	6600
IgM	4300	4500	4300	7200
Neutralizing	1500	1900	3600	3400
Intramuscular:				
ELISA IgG	14000	57000	116000	150000
IgA	2200	7000	14600	16000
IgM	6000	5750	6600	6900
Neutralizing	1800	4400	18000	18000

[a] Geometric mean titre (units/ml) of 12 individual specimens collected after each immunization.

With regard to the toxin-neutralizing antibodies, the mean titre increase was considerably higher after a single intramuscular than after a single oral immunization (Table 4). Peak ELISA antitoxin as well as neutralizing titres to the initial immunizations were always found in the day-14 or day-25 samples and in no instance did the titres decrease to the day-0 levels at the time of the second immunization. The responses in the non-lactating women who received cholera B subunit orally did not differ appreciably from the responses in the orally immunized, lactating women.

A second immunization by either route did not result in significantly higher ELISA IgG or IgA (or even IgM) titres than those induced by the initial immunization, except in cases where an intramuscular dose followed an oral dose. Whereas only a few of the women responded with neutralizing antibodies to the initial oral immunization, 5 out of 7 seroconverted after a second oral dose but the titre increase was lower than after an initial or second intramuscular injection. Of the women receiving an initial intramuscular injection, only one responded with higher neutralizing titres after the second compared with the first immunization.

Unlike the response after the initial immunization, peak titres were often observed already on day 7 after the second immunization. In most cases the peak level titres persisted for at least 4 weeks after the second dose and in only one case did the antitoxin response decrease to the preimmunization levels within this period. However, only 1 woman out of 16 had higher antitoxin titres in the serum after 15 months, compared with the level at the onset of the initial immunization. The third oral immunization resulted in significant antitoxin responses of similar magnitude as after the initial oral immunization. However, whereas peak responses were not observed until 2–4 weeks after the initial immunization, they were usually found already on day 3 after the third immunization (data not shown).

Both to ascertain the purity of the cholera B subunit preparation used and to test whether the titre increases observed were not due to nonsymptomatic cholera infection during the study period, pre- and postimmunization sera (day-0 and day-14 samples) from all vaccinees were tested in parallel against purified cholera B subunit, A subunit, and *V. cholerae* lipopolysaccharide. The titre increases against the purified B subunit were very similar to those seen against whole cholera toxin, while none of the vaccinees responded with a significant titre increase against either purified A subunit or cholera lipopolysaccharide.

Antitoxin responses in saliva and breast milk. Although the total IgA levels in saliva varied 7-fold (range, 20–140 μg/ml) between the different women, the coefficient of variation in total IgA concentration between the different saliva samples from a single person was less than ±10%. Significant, although generally low, IgA antitoxin responses in saliva were found in 50% of the cases after a single oral

Table 5. Number and percentage of significant (i.e., ⩾ 2-fold) antitoxin IgA responses in saliva at different time intervals after first, second and third immunizations with cholera B subunit given orally or intramuscularly

Number of days after immunization	First immunization		Second immunization[a]		Third immunization[b]
	Oral ($n = 12$)	Intramuscular ($n = 12$)	Oral[c] ($n = 5$)	Intramuscular[c] ($n = 4$)	Oral[c] ($n = 16$)
3	—	—	—	—	4 (25)[d]
7–9	2 (17)[d]	5 (42)	2 (40)	2 (50)	5 (31)
14	4 (30)	6 (50)	3 (60)	2 (50)	—
25–28	4 (30)	7 (58)	2 (40)	0	4 (25)
Up to 28	6 (50)	9 (75)	3 (60)	3 (75)	7 (44)

[a] Interval between 1st and 2nd immunization was 25 days, and between 2nd and 3rd immunization was 15 months.

[b] After an initial oral or intramuscular immunization

[c] The third immunization was oral, the preceding two immunizations being two orals, or oral followed by intramuscular, or intramuscular followed by oral, or two intramuscular doses.

[d] Figures in parentheses are percentages

administration and in 75% after the initial intramuscular injection of B subunit (Table 5). The titres (by either route) peaked on day 14 or 25 and were, on average, about 2-fold higher than in the pre-immunization samples. Toxin neutralizing antibodies could not be detected with the adrenal cell test in either the pre- or postimmunization saliva samples. In only one case was there a significant titre increase in saliva that was not paralleled by a significant IgA response in the intestinal lavage specimens.

A second immunization by either route resulted in significant salivary IgA antitoxin responses of about the same frequency and magnitude as was observed after the initial immunizations (Table 5). The oral dose given 15 months after the second immunization also produced a low but significant salivary antitoxin response in about half of the women (Table 5). In some cases the IgA titre rise was observed already in the day-3 saliva specimens.

The possibility of inducing antitoxin IgA antibodies in breast milk by immunization was also studied. Since the total IgA concentrations varied very little in the breast milk from one day to another during the first two months of the study, pre- and postimmunization specimens could be compared without prior adjustment for total IgA. The IgA concentration in breast milk from the different women varied between 0.29 and 0.70 g/l (mean $\pm$ SD = 0.49 $\pm$ 0.17 g/l). Most of the women had substantial IgA antitoxin levels in the milk even before the immunization. A single oral or intramuscular immunization with B subunit resulted in significant IgA titre increases in the milk in only 4 out of 12 and 2 out of 12 cases, respectively, and then the increases were of very low magnitude. A second immunization by either route did not further enhance the response induced by the initial immunization. In each instance, an IgA antitoxin titre increase in breast milk was associated with a significant response in the intestine.

DISCUSSION

The present study shows that a new cholera immunogen, i.e., purified B subunit of cholera toxin, can stimulate significant antitoxin antibody formation both in the serum and locally in the intestine when given either by mouth or by injection. Furthermore, the results suggest that immunization with B subunit may induce an immunological memory that can be boosted after more than one year.

The new immunogen proved entirely safe when given by either route. After oral administration, no side-effects at all were noted. After intramuscular injections, mild side-effects at the injection site were seen in about half of the women but these reactions were considerably fewer and milder than those registered, following the same surveillance protocol, after injection of inactivated whole-cell cholera vaccine (*24*).

Although serum antibodies that leak into the intestine may have some protective effect against cholera (*25*, *26*), locally produced antibodies are probably of greater importance for protection against the disease (*6*, *9*, *27*). Our data suggest that the oral route is superior to parenteral immunization in inducing an effective local antitoxin immune response in the intestine. The magnitude of the mucosal IgA response induced by the two routes did not differ much, but the duration of the response after intra-

muscular injection was distinctly shorter than after the oral dose. These results are in accord with the findings in experimental animals which showed that parenteral immunization with cholera toxoid, in contrast to oral immunization, generally resulted in very transient antitoxin responses in the intestine (*28*, *29*).

The immunization scheme was designed to allow comparison between the following: two oral, oral followed by intramuscular, intramuscular followed by oral, and two intramuscular vaccine administrations; this scheme was based upon initial suggestions from studies in animals by Pierce et al. that cholera toxoid would be poorly effective as a local antigen except when after parenteral immunization (*28*) and the later suggestion that parenteral immunization may suppress the gut mucosal immune response to a subsequently applied antigen by the oral route (*30*). Unlike the results in these animal studies, we found in our human subjects no enhancement or suppression of the response to an oral dose of B subunit by prior intramuscular immunization since the group receiving two oral doses responded similarly to the group receiving an intramuscular and then an oral dose. Species differences and the previous intestinal priming of our volunteers by natural exposure may account for the different results observed. There should therefore be caution in planning immunization regimens for humans based only on data from experimental animals

Unlike the local intestinal response referred to above, the ELISA antitoxin as well as neutralizing antibodies in serum were more effectively induced by parenteral than oral immunization. Leakage of serum antibodies into the intestine appeared to be practically insignificant. This is supported by our findings of relatively low levels of total IgG as opposed to total IgA in intestinal lavage fluid and to the lack of a relation between IgG antibody responses in serum and lavage fluid. Furthermore, 71–86% of the IgA antitoxin in lavage fluid was shown by affinity chromatography to be SIgA.

Cholera disease has been shown to give almost solid protection against rechallenge for at least three years after infection in North American volunteers (*1*), and to protect against second attacks for several years in endemic areas too (*2*). Against this background, our finding that mucosal antitoxin levels decreased from day 14 to day 25 in many cases after oral immunization and that only 1 out of 16 women had elevated antitoxin titres in the intestine 15 months after the initial immunization could be interpreted as suggesting that the vaccine was relatively inefficient in inducing a local immune response. However, both the magnitude and the duration of the antitoxin antibody responses induced by oral immunization with cholera B subunit were comparable to those observed in convalescents from severe clinical cholera in Bangladesh (*11*, *24*). Therefore, we are inclined to believe that the long protection observed after clinical disease might more probably be due to the stimulation of a local immunological memory that may be rapidly boosted by renewed contact with cholera antigen before it gives rise to disease. Therefore, we tried to examine whether the two initial immunizations with B subunit could have induced an immunological memory in the intestine. Studies in animals have demonstrated the existence of a long-lasting mucosal memory after immunization with cholera toxin or toxoid (*6*, *28*, *30*).

Since this study was conducted in an area endemic for cholera as well as enterotoxin-producing *Escherichia coli*, many of the vaccinees could have previously been exposed to one or more enterotoxin antigens by natural infection. This is supported by the finding of significant antitoxin levels in most of the women both in serum and in the intestine at the onset of B subunit immunization. A second or a third immunization with B subunit did not result in any higher antitoxin levels in the intestine and serum than did the initial oral administration of B subunit. However, elevated antitoxin antibody levels in the intestine were observed already on day 3 after the third (oral) immunization in more than half of the cases, which is significantly earlier than after an initial oral immunization with B subunit, as tested concomitantly in other women from the same village (*24*). This suggests that the initial immunizations induced an immunological memory in the intestine that could be boosted even 15 months later, the response being characterized by a rapid onset rather than high peak antibody titres. A similar early "memory" response was noted in a parallel study after a second oral dose of cholera B subunit given 4 weeks after an initial oral B subunit immunization (*24*).

Determination of antibodies in the intestinal lavage fluid seems to be a useful approach to measuring intestinal immune responses, as shown both by this study and others (*11*, *24*). The lavage fluid contains predominantly antibodies of the SIgA type (i.e., antibodies that have been locally produced) and, unlike jejunal aspirates, it contains immunoglobulin derived from the entire length of the small intestine. However, the method is relatively time-consuming and it is therefore important to find simpler approaches for measuring local cholera responses, especially one that could be used in the field. On the basis of findings in experimental animals that suggested the existence of a common mucosal immune system with significant communication from one site to the others (*31*, *32*), we studied the intestinal immune responses as well as the responses in breast milk and saliva. Frequent antibody titre rises in saliva were noted that corresponded with titre rises in the intestine. The results suggest that

antibody determinations in saliva may be useful as a proxy measure of the intestinal immune responses. This hypothesis is now being investigated in a more elaborate study with more data points (Jertborn et al., in preparation).

We conclude that oral immunization with purified cholera B subunit is entirely safe, and gives rise to gut mucosal antitoxin responses and immunologic memory comparable to those induced by cholera disease. The promising features of B subunit make it a candidate immunogen to be included in an oral cholera vaccine together with vaccine components inducing mucosal antibacterial immunity that could cooperate synergistically with the antitoxic antibodies in providing immune protection against cholera. Such a vaccine has been prepared and found to be protective in volunteers against challenge with a high dose of live *V. cholerae* organisms (Levine, M.M., and Black, R.E. et al., in preparation).

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RÉSUMÉ

APPARITION LOCALISÉE ET GÉNÉRALISÉE D'ANTICORPS ET D'UNE MÉMOIRE IMMUNOLOGIQUE CHEZ L'HOMME APRÈS DIFFÉRENTS MODES DE VACCINATION AVEC LA SOUS-UNITÉ B DE LA TOXINE CHOLÉRIQUE

Une étude a été réalisée afin d'évaluer l'aptitude d'une nouvelle substance immunogène cholérique, la sous-unité B purifiée de la toxine cholérique administrée par voie orale et intramusculaire, à susciter une réponse anticorpale locale ou générale et à faire apparaître une mémoire immunologique au niveau de la muqueuse intestinale chez 30 femmes Bangladeshi allaitantes ou non. Une vaccination orale ou intramusculaire a entraîné chez 10 sujets sur 11 et chez 9 sujets sur 11 respectivement, une production significative d'immunoglobulines A (IgA) sécretoires anti-toxine dans l'intestin comme l'a montré un turage de type ELISA. L'augmentation du titre d'IgA dans le liquide de lavement intestinal était le même pour les deux modes de vaccination, mais la réponse était généralement plus durable en cas de vaccination par voie orale. Une deuxième vaccination orale ou intramusculaire pratiquée au bout de 25 jours n'a pas entraîné de réponse plus importante que la première vaccination. Alors que, chez 9 des 15 sujets, les taux d'antitoxines étaient sensiblement plus élevés quatre semaines après la première ou la deuxième vaccination, seul un sujet sur 16 présentait encore un titre élevé d'antitoxines intestinales au bout de 15 mois. Une troisième vaccination orale, pratiquée au bout de 15 mois, a produit dans l'intestin, en intensité comme en fréquence, les mêmes réactions que la première vaccination orale. Toutefois, après la troisième vaccination, on observe une réponse immunitaire sensible dès le troisième jour chez la majorité des sujets (9 sur 15 contre 1 sur 16 après la première vaccination, $P<0,05$), ce qui suggère l'acquisition d'une mémoire immunologique au niveau de la muqueuse intestinale à la suite de la première vaccination. La première vaccination buccale ou intramusculaire entraîne une augmentation sensible du titre des antitoxines sériques chez la plupart des sujets (11 sur 12). En ce qui concerne l'intensité des réponses, on constate que c'est la réponse en anticorps anti-toxine qui est de loin la plus importante dans le cas de la vaccination intramusculaire. Une deuxième ou une troisième vaccination n'entraîne pas de réponse plus marquée sauf si l'on pratique une injection intramusculaire après la vaccination buccale. Alors qu'après la première vaccination les titres maximaux étaient toujours observés le 14e ou le 25e jour, ils atteignent souvent leur maximum au bout de 7 jours seulement après la deuxième vaccination et dès le troisième jour après la troisième vaccination. Une production sensible d'IgA était observée au niveau salivaire dans environ 50% des cas après vaccination par voie orale et dans 75% des cas après une ou deux vaccinations intramusculaires alors que le taire de l'IgA dans le lait n'était augmenté que chez 6 des 24 femmes allaitantes

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